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Autoradiographic studies of solute transport across the toad bladder

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Autoradiographic studies of solute transport across the toad bladder. The autoradiography of diffusible, hydrophilic solutes presents special problems in localization of the labeled solute under study. We present studies of the movement of ^{14}C-labeled urea, and ^{14}C- and ^{3}H-labeled sucrose across the isolated urinary bladder of the toad, a vasopressin-sensitive epithelium, using a technique that avoids exposure to water throughout all processing steps and minimizes error caused by isotope scatter. We have shown a significant increase in ^{14}C urea entry into epithelial cells following vasopressin, and a significant decrease following phloretin, an agent that selectively blocks vasopressin-stimulated urea transport. The autoradiographic technique confirms the luminal site of action of phloretin. Studies of ^{14}C and ^{3}H sucrose labeling show that this molecule is virtually excluded from the cell. The current method of grain counting is capable of yielding reliable information in studies of epithelial transport.

Etudes autoradiographiques du transport de solutés à travers la vessie de crapaud. L'autoradiographie de solutés diffusibles et hydrophiles pose des problèmes spéciaux de localisation du soluté marqué étudié. Nous présentons des études du mouvement de l'urée marquée au ^{14}C, et du sucrose marqué au ^{14}C et au ^{3}H à travers la vessie de crapaud isolée, un épithélium sensible à la vasopressine, à l'aide d'une technique qui évite l'exposition à l'eau pendant toutes les étapes et minimise l'erreur due à la dispersion de l'isotope. Nous avons montré une augmentation significative de l'entrée de ^{14}C urée dans les cellules épithéliales après vasopressine, et une diminution significative après phlorétine, un agent qui bloque sélectivement le transport de l'urée stimulé par la vasopressine. La technique autoradiographique confirme le site d'action luminal de la phlorétine. Des études de marquage du sucrose du ^{14}C et au ^{3}H montrent que cette molécule est virtuellement exclue de la cellule. La méthode habituelle de comptage de grains peut fournir une information fiable dans les études de transport épithélial.

In theory, autoradiography offers an ideal method for the study of solute transport across epithelia. A great number of molecules, labeled with isotopes of low energy, can be used, and their position in the cell under a variety of experimental conditions can be determined. However, serious problems are encountered if one wishes to study water-soluble, diffusible solutes by autoradiography. One cannot use conventional methods of specimen preparation, since slow fixation, multiple washings and extractions, and other steps make it impossible to maintain the exact position of the labeled molecule within the tissue.

Several techniques, involving rapid freezing as the first step, have been developed for the autoradiography of diffusible solutes [1–4]. In 1967, Stirling and Kinter [5] reported a method in which the tissue was frozen rapidly, freeze-dried, and embedded in epon. Sections were cut, floated off the knife in water, and covered with emulsion; the autoradiograph was developed after a suitable time interval. Figure 1 shows a toad bladder prepared by the Stirling and Kinter technique. While this method provided excellent morphology, the exposure to water interfered with solute localization, and satisfactory quantitative data could not be obtained. The Stirling and Kinter method was modified by Masland and Mills [6], who sectioned and processed their material in the total absence of water, giving more accurate solute localization, especially for substances not bound to a cellular component. We used the Masland and Mills technique to demonstrate the labeling of toad bladder epithelial cells by ^{14}C urea and modified the method further to reduce scatter, giving more accurate estimates of intracellular localization of labeled solutes.

The experiments reported in this paper are based on these recent modifications and are presented to demonstrate the potential importance of autoradiography in the study of epithelial transport, even in an epithelium as thin as the toad bladder.

Methods

Masland-Mills method. Hemibladders from female Dominican toads *(Bufo marinus)* were mounted and tied to the ends of 1-cm diameter polypropylene rings, with the luminal surface facing outward. Approximately two such preparations were made from each bladder. Amphibian Ringer's solution (0.5 ml: 120 mM Na^+, 4.0 mM K^+, 0.5 mM Ca^{++}, 116 mM Cl^-, 5 mM phosphate, pH 7.4, 230 mOsm/kg), was placed inside the ring, bathing the serosal surface. A 15-min period of preincubation was carried out in which the luminal surfaces of the rings were bathed in 20 ml of Ringer's solution. In the phloretin experiments 10^{-4} M phloretin (K & K Laboratories, Inc., Plainview, New York, USA) in 0.5% ethanol was present in the luminal solution of the experimental bladder halves; 0.5% ethanol alone was present in the luminal solution of the control bladder halves. In these experiments, vasopressin was present in the

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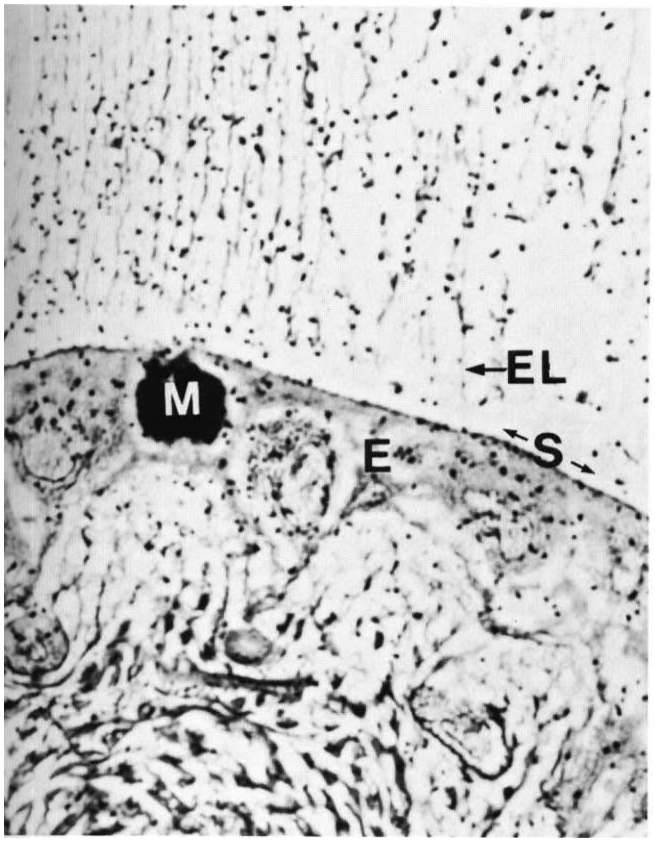


Fig. 1. *Autoradiograph obtained by the Stirling-Kinter method of an 0.8-μm thick section of a control bladder incubated for 5 min in an isotonic luminal bathing medium containing 50 μCi/ml of ^{14}C urea and 10% bovine serum albumin.* The epithelial cells (**E**) are clearly shown. Most are granular cells; a mucus cell (**M**) is also present. Grains are seen in the epithelial cells, and grains in the luminal bathing medium are distributed linearly along eutectic lines (**EL**). **S** represents a strip free of eutectic lines. (×900)

serosal bathing medium of both the experimental and control bladder halves.

After preincubation, the luminal surfaces of the rings were placed carefully in 1.0 ml of Ringer's containing the isotopically labeled molecule to be studied (^{14}C urea and ^{14}C sucrose at 50 μCi/ml; ^{3}H sucrose at 100 μCi/ml, New England Nuclear Corp., Boston, Massachusetts, USA). In the phloretin experiments, the Ringer's solution was diluted 1:5 with distilled water to swell the epithelial cells, increasing their volume, and reducing the problem of isotope scatter. The time of exposure to isotopes was 15 sec. Permeability coefficients were determined by sampling the serosal and mucosal media just prior to freezing. These coefficients were approximate, in view of the short time period involved, but did permit validation of drug effects.

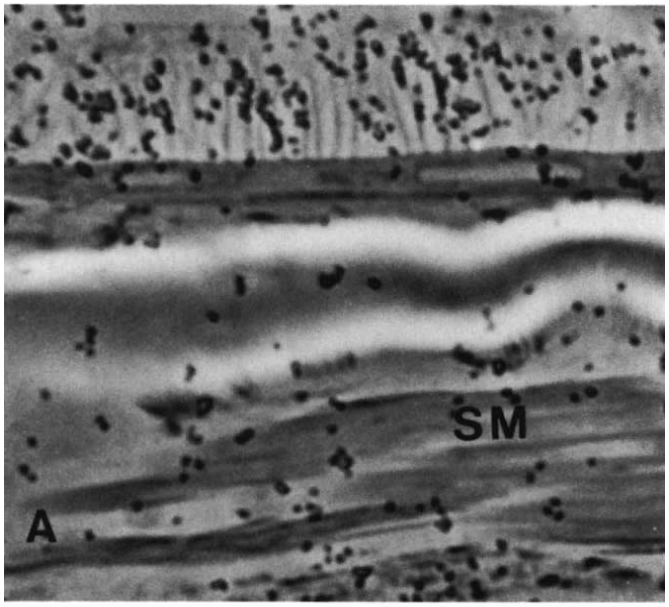
The diluted Ringer's solution that bathed the luminal surface also contained 7 to 10% bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri, USA). The albumin, as well as other solutes, is excluded from the ice phase upon freezing [7, 8] and, when the ice is evaporated during freeze-drying, forms a fine linear network of eutectic lines to which the isotopically labeled solutes are bound [5, 8]. This method of producing eutectic lines permits the determination of isotope concentration in the luminal bathing medium in each individual section, and thus permits the estimation of cell-to-medium ratios of the labeled solute.

At the end of the isotope incubation period, the serosal solution was discarded rapidly and the ring was plunged into liquid propane at −170°C for 30 sec. The ring was stored in liquid nitrogen, then introduced into a freeze-dry appartus (Redi Industries, Hempstead, New York, USA) under a dry nitrogen atmosphere. The freeze-dryer was of the cryosorption type originally designed by Stumpf and Roth [9]. Freeze-drying was carried out for 18 hr. The specimens were then returned to room temperature over a period of 3 hr. Two to three crystals of osmium tetroxide were introduced quickly via the ionization tube cap, and the tissue samples were exposed to osmium vapor for 2 hr. The specimens were removed under dry nitrogen and placed in degassed liquid Spurr (Polysciences, Inc., Warrington, Pennsylvania, USA) for a 30-min impregnation. This short time proved to be sufficient for these thin tissue samples. The samples were then cut into small sections with a razor blade, placed in Spurr in silicone molds and cured at 60°C for 18 to 24 hr. After trimming, the blocks were placed in a Sorvall MT2B ultramicrotome, and 1 to 2 μm sections were cut with dry glass knives. Dry sections were picked off the knife with a 26-gauge needle, placed on dry clean slides, and stored in a dessicator prior to transfer to emulsion-coated slides. The slides had been cleaned carefully in acid, detergent, water and alcohol and then dried. Then, in a darkroom, they were dipped in emulsion (Kodak NTB-2) which had been melted at 43°C. The slides were stored in a light-tight box with dessicant until needed.

Sections were transferred in the dark to the emulsion under completely dry conditions by gently pressing the slides on which they had been placed against the emulsion-coated slides. The completed slides were stored at −20°C in light-tight boxes with dessicant for a period of exposure of 2 to 3 days for ^{14}C and 5 to 7 days for ^{3}H.

Grain counting. Experiments were carried out with coded slides so that the observer was unaware of whether a given slide was from an experimental or control bladder. Estimates of epithelial cell and luminal medium area were made with a camera lucida (Zeiss) attachment. The outlines of both areas were traced on paper, and all of the grains within the outlines were counted. The epithelial and luminal areas were determined with an electronic graphics calculator (Numonics Corporation, Lansdale, Pennsylvania, USA). At least 200 (and usually 500 to 1000) epithelial grains, and 1,000 luminal grains were counted in each slide; grain counts were expressed as counts per cm^2 of camera lucida drawing, and the cell-to-medium ratio was determined. Correction for grains in the emulsion itself was made by counting grains in an area far removed from the section; emulsion grains were approximately 1 per cm^2, and this value was subtracted from cell and medium counts.

Our major modification of the method, which resulted in significantly lower intracellular counts, was to count intracellular grains in those regions where no luminal eutectic lines were present (see Fig. 4). This resulted in a lowering of intracellular counts by approximately 50%, compared to counts in cells adjoining regions where eutectic lines were present, showing that scatter from eutectic grains was being counted in our earlier experiments as being present in cells. Medium



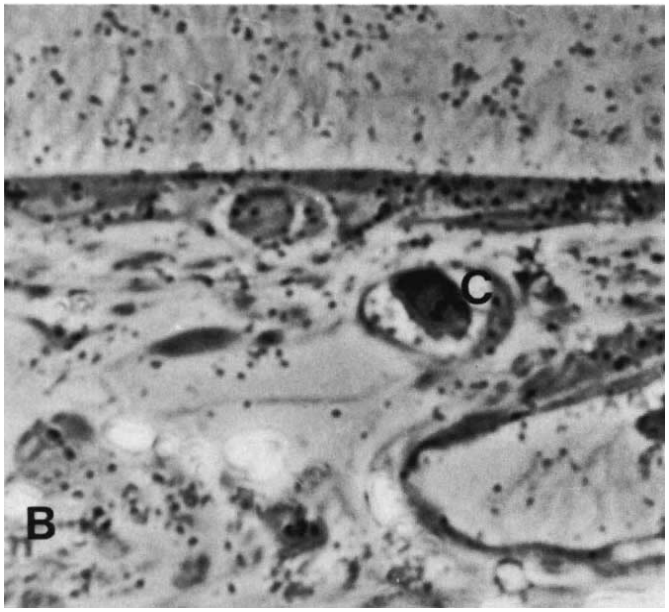


Fig. 2. *Autoradiographs (Masland-Mills method) of toad bladders in the absence* **(A)** *and presence* **(B)** *of vasopressin.* Section thickness was 2 μm in this and in Figures 3 to 5. Bladders were incubated for 1 min with 50 μCi/ml of ^{14}C urea and 10% bovine serum albumin in the luminal bathing medium. The luminal Ringer's solution was isotonic in this series of experiments. Eutectic lines and their associated grains are again seen in the luminal bathing medium; grain density in the epithelial cells is less in the control preparation than in the vasopressin-treated preparation. In the supporting layer, smooth muscle **(SM)** and capillaries **(C)** are seen. (×1220)

counts, of course, were determined from the eutectic layr as before.

The significance of the results was determined in the ^{14}C urea experiments by pair analysis, using Student's *t* Test [10]. Unpaired analysis was used in comparing ^{14}C and ^{3}H sucrose results.

Table 1. Cell-to-medium ratios for ^{14}C urea in the presence and absence of vasopressin

Vasopressin	c/m ratio ± SEM (5 paired experiments)
Absent	0.57 ± 0.03
Present	1.06 ± 0.10
	Δ0.49
	$P < 0.01$

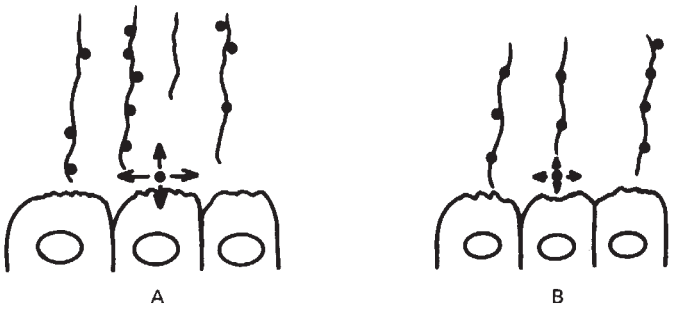


Fig. 3. *Scatter of ^{14}C and ^{3}H isotopes from their true locations on eutectic lines in the luminal bathing medium.* ^{14}C has a wide enough radius of scatter to develop a grain over the epithelial layer; ^{3}H is less likely to be counted in the epithelial layer.

Results

Figures 2 **A** and **B**, prepared from an earlier study [11], show autoradiographs of toad bladders exposed to ^{14}C urea in the luminal bathing medium, in the absence **(A)** and presence **(B)** of vasopressin. The sections in these and in the following figures are thicker (2 μm) than in the Stirling-Kinter sections, but good morphology is preserved. There are numerous grains in the luminal medium, bound to eutectic lines formed by the albumin, which forms a fine network when the ice is evaporated during freeze-drying. By counting the density of grains in a given area, we obtained an index of the concentration of isotope in the medium. There are grains in the epithelial cells as well; they are more abundant in the vasopressin-treated bladder. Their density per unit area was also determined; Table 1 shows the cell-to-medium ratios in 5 paired experiments. There was a significant increase in the cell-to-medium ratio following vasopressin. In this early series, however, intracellular labeling by ^{14}C urea was high in both the control and hormone-treated preparation, considerably higher than the 11 and 38% labeling pre- and post-vasopressin, obtained by Maffly et al [12] in experiments with homogenized bladders. We were able to show that the high intracellular labeling was due in large part to scatter from ^{14}C grains on the eutectic lines in the medium near the cells. This is shown diagramatically in Figure 3. Here we see that a ^{14}C grain in the medium near the epithelial cell has enough scatter to be recorded incorrectly as being within the cell. One would not expect this problem with a less energetic isotope such as tritium, shown on the right of the figure.

To deal with the problem of scatter of ^{14}C, we modified our method of counting ^{14}C grains, as shown in Figure 4. This figure again shows ^{14}C urea grains in the medium and in the epithelial cells. In most sections, there are areas where the eutectic lines

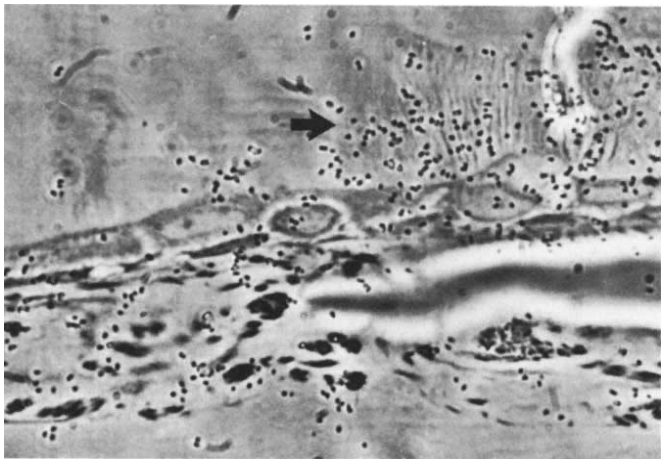


Fig. 4. *Autoradiograph of a toad bladder treated with vasopressin in the presence of an osmotic gradient, and incubated for 15 sec with 50 μCi/ml of ^{14}C urea and 7% bovine serum albumin in the luminal bathing medium.* The figure shows the difference in intracellular grain density in the presence and absence of a luminal eutectic layer. On the *right*, there is a eutectic layer, which stops abruptly at the *arrow* (note that the layer is slightly out of focus near the *arrow*, but lines are still discernible). Numerous grains are present in both the eutectic layer and the epithelial cells below. On the *left* of the figure there are few grains in the luminal area, and a reduced number in the epithelial cells. (×1000)

Table 2. Cell-to-medium ratios for ^{14}C and ^{3}H sucrose

Isotope	c/m ratio ± SEM	
	Eutectic layer *Present*	Eutectic layer *Absent*
^{14}C	0.23 ± 0.03 (13)[a]	0.09 ± 0.01 (12)
^{3}H	0.08 ± 0.02 (11)	0.08 ± 0.02 (6)
P (^{14}C vs. ^{3}H)	<0.001	NS

[a] Figures in parentheses refer to the total number of fields counted.

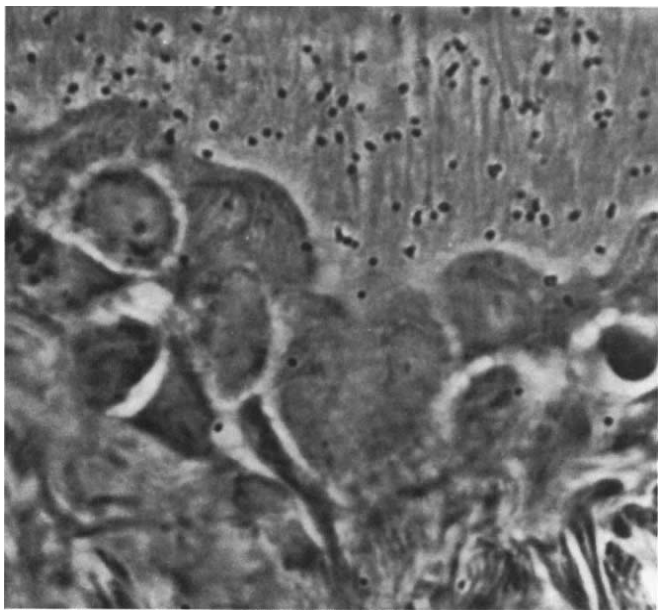


Fig. 5. *Distribution of ^{3}H-labeled sucrose.* (×2500)

Table 3. Effect of phloretin on cell-to-medium ratio of ^{14}C urea

Conditions		c/m ratio ± SEM	
		Eutectic layer *Present* (5 paired expts)	Eutectic layer *Absent* (4 paired expts)
Vasopressin		0.97 ± 0.10	0.55 ± 0.07
Vasopressin plus 10^{-4} M phloretin		0.68 ± 0.09	0.36 ± 0.06
		Δ0.29 ± 0.09	Δ0.19 ± 0.04
	P	<0.05	<0.025

are absent, as in the left of the figure. There are fewer grains in the epithelial cells in this area since scatter from grains in the medium is not possible. By counting intracellular grains in these areas, and comparing their density to grains in the eutectic areas, it should be possible to obtain more valid cell-to-medium ratios.

We tested this method by determining cell-to-medium ratios for ^{14}C and tritiated sucrose. The epithelial cells are virtually impermeable to sucrose, and the cell-to-medium ratio should be close to zero. Table 2 shows cell-to-medium ratios for ^{14}C and tritium-labeled sucrose, determined in areas containing and areas free of eutectic lines. The ^{14}C ratio was 0.23 in areas containing eutectic lines and 0.09 in areas without them. The tritium ratio was the same with or without eutectic lines, as would be expected with the weaker isotope. Figure 5 shows the distribution of tritiated sucrose in an actual experiment. It can be seen that the sucrose remains totally outside the cells.

We finally determined cell labeling by ^{14}C urea in paired bladders, both of which were treated with vasopressin, and one of which was also exposed to 10^{-4} M phloretin in the luminal medium. Phloretin inhibits urea transport without interfering with osmotic water flow [13]. It is believed to act at the luminal entry site for urea, but direct evidence is lacking. We carried out a series of paired experiments, again in a blind fashion. Table 3 shows that the cell-to-medium ratio for ^{14}C urea entering from the mucosal medium was 0.97 following vasopressin, and fell significantly to 0.68 when phloretin was present. When we corrected for luminal scatter, the intracellular counts as a whole were significantly lower, and the cell-to-medium ratio again showed a significant fall following phloretin (0.55 to 0.36, $P < 0.025$). In all of the five paired bladders, there was the expected reduction in urea permeability in the presence of phloretin, from 301 ± 83 to 105 ± 34 × 10^{-7} cm· sec (Δ = 196 ± 75 SEM; $P = 0.06$).

Discussion

Autoradiography offers one of the more satisfactory solutions to the problem of solute localization in transporting epithelia. There is no limit to the variety of solutes that can be studied, as long as they can be labeled with an isotope of relatively low energy. However, as already discussed, localization of water-soluble, diffusible solutes, which are not tightly bound or incorporated into larger molecules, presents problems of a

special kind. By using a rapid-freeze, freeze-dry method, and avoiding any contact with water in the later stages of sectioning and contact with emulsion, we have verified by autoradiography some existing observations regarding urea transport and sucrose exclusion in the toad bladder. While some movement (leaching) of water-soluble label into the embedding plastic could in theory take place, and, indeed, has been observed with methods different from ours [2], our sections show that leaching is not a major source of error. For example, the zone of clear plastic above and also the left of the eutectic area in Figure 4 is almost free of grains, showing minimal migration of the ^{14}C urea away from the euctectic lines. The same is true of areas free of eutectic lines in Figure 1. Leaching was not observed in the earlier studies of Stirling and Kinter [5]. Osmium vapor represents another potential source of solute translocation; we have been unable to study this as an independent source of error, but we have been reassured by the results of the sucrose labeling experiments, where the predicted exclusion of label from cells was observed.

For ^{14}C sucrose, the cell-to-medium ratio was less than 0.10 when corrected for scatter, a value virtually identical to that for ^{3}H sucrose. Of the small number of grains in the epithelial layer, some undoubtedly were crossing the epithelium by a paracellular route.

The studies with phloretin again confirm earlier observations, in which 10^{-4} M phloretin selectively inhibited vasopressin-stimulated urea transport across the toad bladder [13]. These studies provided some of the evidence for the view that water and solutes moved across the rate-limiting luminal cell membrane via independent pathways, and that both types of pathways were influenced by vasopressin [14, 15]. While it was assumed that phloretin (the aglucone of phlorizin) blocked the luminal transport sites for urea, there was no direct evidence for this site of action, as opposed to a site at the basolateral cell membrane or some intermediate barrier. Autoradiography has provided this evidence, showing that urea entry across the luminal barrier was significantly decreased by phloretin. In addition, our studies show that the large majority of epithelial cells are involved in urea transport, since virtually all cells appeared to contain ^{14}C urea. Since granular cells make up approximately 80% of the total cell population, it would appear to be the granular cell that is involved in urea, as well as water transport. We have the impression from the pattern of cell labeling that a transcellular, rather than a paracellular pathway is involved in urea transport; here, however, it will be necessary to refine our technique further, using thinner sections and a more complex statistical analysis to establish this point, since the method we have used thus far is best suited to distinguishing extracellular versus intracellular grains.

Our value of 0.55 for cell labeling in the presence of vasopressin alone is close to the older value of 0.38 obtained by blotting and homogenizing vasopressin-treated bladders labeled with ^{14}C urea from the luminal bathing medium [12]. There were uncertainties in this earlier study regarding the role of the relatively large serosal inulin space in influencing intracellular counts; correction for this space would increase estimated cell labeling substantially. In this respect, we regard our autoradiographic value as a better index of true intracellular labeling.

Our goal in carrying out these experiments, however, was not primarily to verify earlier studies, but to demonstrate the potential of the "dry" technique of autoradiography to disclose details of solute transport not readily obtainable by other methods. Localization of solute position is good enough to determine at the light microscopic level which cells in a transporting epithelium are involved in the transport process, and the site of action of hormones and other agents that influence solute transport.

Acknowledgments

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